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A novel mechanism for glucose side-chain formation in rhamnose-glucose polysaccharide synthesis[1]

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Abstract **We have cloned two genes (*rgpH* and *rgpI*) that encode proteins for the formation of the glucose side-chains of the *Streptococcus mutans* rhamnose-glucose polysaccharide (RGP), which consists of a rhamnan backbone with glucose side-chains. The roles of *rgpH* and *rgpI* were evaluated in a rhamnan-synthesizing *Escherichia coli*. An *E. coli* strain that harbored *rgpH* reacted with antiserum directed against complete RGP, whereas the *E. coli* strain that carried *rgpI* did not react with this antiserum. Although *E. coli:rgpH* reacted strongly with rhamnan-specific antiserum, co-transformation of this strain with *rgpI* increased the number of glucose side-chains and decreased immunoreactivity with the rhamnan-specific antiserum significantly. These results suggest that two genes are involved in side-chain formation during *S. mutans* RGP synthesis in *E. coli*: one gene encodes a glucosyltransferase, and the other gene probably controls the frequency of branching. This is the first report to identify a gene that is involved in regulation of branching frequency in polysaccharide synthesis.**

Key words: Glucosyltransferase; Glucose side-chain formation; Regulation; Rhamnose-glucose polysaccharide; *Streptococcus mutans*

1. Introduction

The cariogenic bacterium *Streptococcus mutans* produces rhamnose-glucose polysaccharides (RGPs), which are composed of α1,2- and α1,3-linked rhamnan backbones and glucose side-chains that are linked to alternate rhamnoses in the cell wall [1,2]. Previously, we identified the *rgpE* gene, which is involved in glucose side-chain formation, by inactivation of the gene in *S. mutans* [3]. In addition, we characterized five genes (*rgpA*, *B*, *C*, *D*, and *F*) that participate in the rhamnan backbone assembly, by gene inactivation in *S. mutans* and expression of the genes in *Escherichia coli*, which was based on similarities in RGP synthesis between *S. mutans* and *E. coli* [4]. Although these six *rgp* genes appear to be sufficient for RGP assembly in *E. coli*, an *E. coli* strain that harbored these six *rgp* genes was able to synthesize the rhamnan backbone linked to the lipid A core in lipopolysaccharide (LPS) as an O polysaccharide, but not the glucose side-chains [4]. To the best of our knowledge, glycosyl transfer during polysaccharide synthesis is catalyzed by a single glycosyltransferase, and the donor- and acceptor-specificities of the enzymes determine both the nature of the glycosidic linkage and the product structures. On the basis of our previous findings, it appears that glucose side-chain formation on the RGP of *S. mutans* is catalyzed by an unknown mechanism.

In this study, we sought to identify additional genes for glucose side-chain formation during RGP synthesis, and we analyzed the functions of the cloned genes in *E. coli*. In the course of this study, we discovered a novel gene that regulates the frequency of side-chains and a gene that encodes a glucosyltransferase isoform.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The *S. mutans* and *E. coli* strains and plasmids generated in this study are listed in Table 1. The *S. mutans* strains (Xc, Xc45, and Xc47) and *E. coli* strains (DH5α and CS2775) were obtained as described previously [3–5]. Strains of *S. mutans* and *E. coli* were maintained and grown routinely as described previously [6]. Antibiotics were used at the following concentrations: 300 μg/ml erythromycin, 50 μg/ml ampicillin, 25 μg/ml kanamycin, or 20 μg/ml chloramphenicol for *E. coli*; 10 μg/ml erythromycin or 50 μg/ml kanamycin for *S. mutans*.

2.2. DNA manipulations

Standard DNA recombinant procedures, such as DNA isolation, endonuclease restriction, ligation, and agarose gel electrophoresis, were carried out as described by Sambrook and Russell [5]. Transformation of *S. mutans* and *E. coli* was carried out as described previously [6]. The nucleotide sequences were determined with a 373 Stretch automated sequencer (PE Applied Biosystems) as described previously [6]. The DNASIS software (Hitachi Software Engineering Co.) was used for sequence analysis. Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously [7].

2.3. Colony blotting

Transformed bacterial colonies were transferred directly from agar plates onto dry nitrocellulose filters and lysed as described by Meyer et al. [8]. Blotting was performed according to the methods of Hawkes et al. [9].

2.4. Immunodiffusion analysis

Autoclaved extracts from whole cells of *S. mutans* and *E. coli* strains were used for immunodiffusion analyses with the serotype c-specific antiserum which recognizes the α1,3-linked glucose side-chain on the α1,2- and α1,3-linked rhamnan backbone and rhamnan-spe-

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[1] Dedicated to the memory of Prof. Toshihiko Koga, our mentor.
[2] Deceased.

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cific antiserum which recognizes the α1,2- and α1,3-linked rhamnan backbone. These antisera were prepared in our laboratory as described previously [3,10]. Immunodiffusion was performed in 1% (w/v) Noble agar in phosphate-buffered saline (PBS, pH 7.3) [11].

2.5. LPS analysis by ELISA

The immunogenicity of RGP produced in the *E. coli* transformants was examined by enzyme-linked immunosorbent assay (ELISA). LPS was extracted from lyophilized *E. coli* cells using the hot phenol–water extraction procedure as described previously [4]. LPS was suspended in PBS (pH 7.4) at a concentration of 10 mg/ml. Each well of a high-binding microtiter plate (Costar 3590, Corning) was coated with 100 μl of a serial dilution of LPS in PBS. After incubation at 4°C for 20 h, the plate was washed three times with PBS containing 0.05% Tween 20, and blocked at room temperature for 4 h with PBS–0.05% Tween 20 containing 1% bovine serum albumin. The coated plate was incubated at 37°C for 1 h with a 1:500 dilution in PBS of either the serotype c-specific antiserum or rhamnan-specific antiserum (100 μl per well). Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Zymed) followed by the addition of *p*-nitrophenyl phosphate substrate solution (1 mg/ml). After incubation at 37°C for 30 min, the OD_{405} was measured with a microplate reader (Bio-Rad). The values shown in Table 2 were determined from a linear regression plot of several LPS concentrations, and expressed as OD values per mg of LPS per min of incubation.

2.6. Sugar composition of the LPS and cell walls

LPS was prepared as described above, and detached from lipid A by hydrolysis with 1.0% (v/v) acetic acid at 100°C for 3 h. Non-solubilized lipid A was separated by Folch partition [12], and the supernatant was lyophilized. *S. mutans* cell walls were prepared as previously described [3]. Component sugars of the polysaccharides were analyzed by high-pressure liquid chromatography (HPLC) using 5-μm Lichrospher 100 Diol column (Merck) with evaporative light scattering detector (Sedex-75, Sedere) [13], following hydrolysis in 4 M trifluoroacetic acid at 100°C for 3 h. The eluent used in HPLC was 93% (v/v) acetonitrile in water. The flow rate was 1.0 ml/min and the column was operated at 25°C.

2.7. RNA isolation and RNA dot blotting

Extraction of total RNA from the *E. coli* transformants was performed as described previously [7]. Five-fold serial dilutions of total RNA, starting with 10 μg, were applied to nylon membranes (Immobilon-Ny$^+$ Transfer membrane, Millipore). Hybridization was performed with the probe labeled with digoxigenin (DIG) by PCR as described previously [14]. The hybridized probes were detected with the DIG luminescence detection kit (Roche Applied Science).

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the significance of differences between the groups, and a correction for multiple comparisons was done by means of the Bonferroni method.

2.9. Nucleotide sequence accession number

The 4373-bp nucleotide sequence presented in this paper was submitted to the EMBL/GenBank/DDBJ data bank under the accession number AB091254.

3. Results and discussion

3.1. Cloning and sequencing of the S. mutans rgpH and rgpI genes

In this study, the isolation of the additional genes that are involved in the formation of glucose side-chains of RGP was performed by the introduction of an *S. mutans* genomic library into an *E. coli* strain that was capable of producing the rhamnan. Briefly, a partial *Sau*3AI-digest of the *S. mutans* Xc chromosome was ligated to *Bam*HI-digested pMBLcos. This library was transformed into *E. coli* DH5α harboring pRGP118. Transformants were screened by colony blotting with serotype c-specific antiserum, which recognizes the α1,3-linked glucose side-chain on the rhamnan backbone. Six colonies that reacted with serotype c-specific antiserum were isolated from the 2000 colonies in the library. The autoclaved extract from one (pMBLcos11) of the six transformants reacted with serotype c-specific antiserum in the immunodiffusion test and this plasmid contained an inserted DNA fragment of approximately 42 kb. To localize the gene for glucose side-chain formation, pMBLcos11 was partially digested with *Sau*3AI and ligated to *Bam*HI-digested pHCL118. This library was transformed into the rhamnan-producing *E. coli* DH5α strain harboring the plasmid pRGP1. Five transformants that reacted with serotype c-specific antiserum were selected by colony blotting and immuno-

Table 1
Strains and plasmids generated in this study

Strains or plasmids	Genotype or relevant characteristics	Reference or source
Strains		
E. coli		
KD601	CS2775 carrying pRGP1	This study
KD602	CS2775 carrying pRGP20 and pRGPH	This study
KD603	CS2775 carrying pRGP20 and pRGPI	This study
KD604	CS2775 carrying pRGP1 and pRGPH	This study
KD605	CS2775 carrying pRGP1 and pRGPI	This study
KD606	CS2775 carrying pRGP20 and pRGPHI	This study
KD607	CS2775 carrying pRGP1 and pRGPHI	This study
S. mutans		
Xc48	Em^r; strain Xc carrying P15A replicon and Em^r gene inserted into *rgpH*	This study
Xc49	Em^r; strain Xc carrying P15A replicon and Em^r gene inserted into *rgpI*	This study
Xc50	Em^r; strain Xc carrying P15A replicon and Em^r gene inserted into ORF10	This study
Xc60	Em^r Km^r; Xc47 carrying Km^r gene inserted into *rgpE*	
Plasmids		
pHCL118	Cm^r; pUC118 replaced Ap^r gene with Cm^r gene; cloning vector	This study
pMBLcos	Ap^r; cosmid cloning vector	[21]
pRGP118	pHCL118 containing 8.9-kb *Bst*EII-*Stu*I fragment which includes *rmlD* through *rgpF*	This study
pRGP1	Em^r; marker rescue plasmid of 10.0-kb *Bst*EII fragment of Xc47 chromosomal DNA which includes *rmlD* through *rgpF*	[4]
pRGP20	Em^r; marker rescue plasmid of 11.0-kb *Bst*EII fragment of Xc60 chromosomal DNA	This study
pRGPH	pBluescriptII KS$^+$ containing 1.3-kb PCR fragment of the *rgpH* gene	This study
pRGPI	pBluescriptII KS$^+$ containing 0.9-kb PCR fragment of the *rgpI* gene	This study
pRGPHI	pBluescriptII KS$^+$ containing 2.2-kb PCR fragment of the *rgpHI* gene	This study

Table 2
Reactivity of LPSs produced in each *E. coli* transformant with serotype c-specific antiserum and rhamnan-specific antiserum[a]

Strain *rgp* gene(s)[b] in addition to *rgpABCDF*	Reactivity with serotype c-specific antiserum	Reactivity with rhamnan-specific antiserum	Ratio[c]
KD601 *gpE*	ND[d]	19.3 ± 0.21	–
KD602 *rgpH*	18.7 ± 0.08	6.2 ± 0.12	0.33
KD603 *rgpI*	ND	19.1 ± 0.26	–
KD604 *rgpEH*	18.5 ± 0.08	6.2 ± 0.16	0.34
KD605 *rgpEI*	ND	19.0 ± 0.25	–
KD606 *rgpHI*	21.6 ± 0.24	3.2 ± 0.12	0.15
KD607 *rgpEHI*	22.0 ± 0.16	3.1 ± 0.09	0.14

[a]Values are means and standard deviations from three experiments.
[b]Details of genotypes are presented in Table 1.
[c]Ratio was calculated as the proportion of the reactivity with rhamnan-specific antiserum to that with serotype c-specific antiserum.
[d]ND, not detected.

diffusion, in the manner described above. The five clones were designated pGT1, pGT2, pGT3, pGT4, and pGT5. Nucleotide sequence analysis of the inserts in the pGT clones revealed that the fragments overlapped (Fig. 1). Two complete open reading frames (ORFs) and two truncated ORFs at the terminal regions were located in the largest clone, pGT1 (Fig. 1). Furthermore, the nucleotide sequence of the first truncated ORF was identical to that of the 3′-end of ORF7, which is located downstream of the *rgp* locus for rhamnan backbone synthesis [3]. These findings indicate that the newly identified locus is involved in glucose side-chain formation on the RGP, and is located immediately downstream of the *rgp* locus. These results were confirmed using BLAST analysis of the *S. mutans* UA159 genome sequencing database, which is based on the results of the Streptococcal Genome Sequencing Project, on the World Wide Web site of the University of Oklahoma's Advanced Center for Genome Technology. Thus, the other three ORFs were designated ORF8, ORF9, and ORF10. To determine the nucleotide sequence of the 3′-end region of ORF10, we designed primer A (5′-ATAATACCCAACCCCAGC-3′), which corresponds to the region downstream of ORF10, based on the above database. A PCR fragment was amplified using the Xc chromosomal DNA as the template, primer A, and primer B (5′-GAAACCAAGAAAAGACCAG-3′), which was derived from the sequence within ORF9, and the RCR product was sequenced. The predicted translational products for ORF8, ORF9, and ORF10 were proteins of 438, 308, and 312 amino acids, respectively.

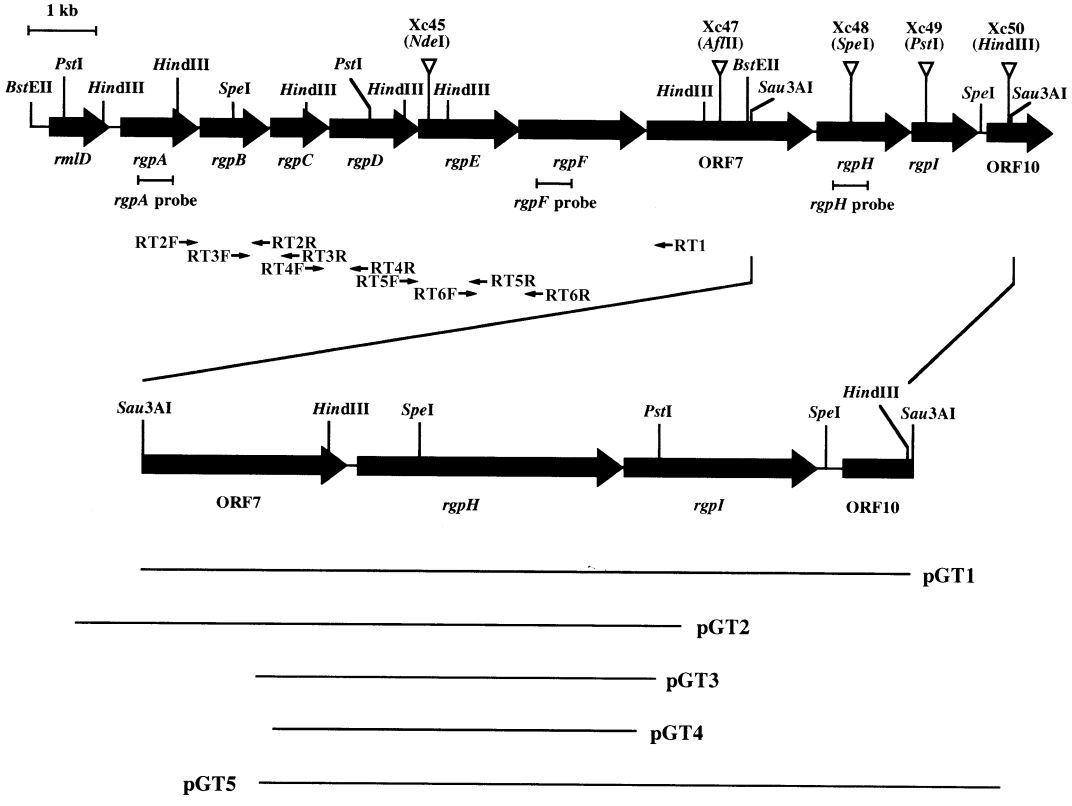

Fig. 1. Restriction map of the *rgp* locus and downstream regions in *S. mutans*. The arrows indicate the locations of the 11 ORFs. The P15A replicon and Em^r gene integration sites for insertional inactivation of each ORF are indicated by inverted open triangles. Relevant primers for RT-PCR are shown by small arrows. The locations of the inserted fragments in pGT1, pGT2, pGT3, pGT4, and pGT5 are indicated in the lower portion of the diagram.

The amino acid sequences deduced from ORF9 and ORF10 had conserved glycosyltransferase domains in their N-terminal portions. On the other hand, the amino acid sequence deduced from ORF8 showed no significant identity with the other proteins. Possible Shine–Dalgarno sequences were identified just upstream of the potential initiation codons of ORF8, ORF9, and ORF10. Consensus −10 and −35 *E. coli* promoter-like sequences (TTGACA-N_{17}-TATGAA) were identified in the region upstream from ORF8.

3.2. Insertional inactivation of ORF8, ORF9, and ORF10

To examine the role of the cloned genes in RGP synthesis by *S. mutans*, ORF8, ORF9, and ORF10 were insertionally inactivated using gene fragments that were interrupted by the P15A replicon and the erythromycin resistance (Em^r) gene at the restriction sites indicated in Fig. 1. The resultant mutants were designated Xc48, Xc49, and Xc50, respectively. Extracts from strain Xc and the mutant strains were analyzed by immunodiffusion with serotype c-specific antiserum. The serotype c-specific antiserum reacted with extracts from strains Xc and Xc50, but not with those from Xc48 and Xc49 (data not shown). Furthermore, the rhamnan-specific antiserum produced precipitin lines with extracts from Xc48 and Xc49 (data not shown). HPLC analysis revealed that the ratio of rhamnose to glucose in the cell wall preparation from Xc50 was 1.96, and the value was similar to that (nearly 2) of the wild-type strain Xc [6]. On the other hand, the glucose content in the cell walls of Xc48 and Xc49 was drastically reduced. These results indicate that, in addition to *rgpE*, ORF8 and ORF9 are involved in glucose side-chain formation on the RGP of *S. mutans* [3]. Therefore, the genes for ORF8 and ORF9 were designated *rgpH* and *rgpI*, respectively.

3.3. Immunological analysis of RGPs from the E. coli transformants

Glucose side-chain formation on the RGP of *S. mutans* requires the *rgpE*, *rgpH*, and *rgpI* genes. Therefore, we evaluated the contributions of *rgpE*, *rgpH*, and *rgpI* to glucose side-chain formation in *E. coli*. Since RGP produced in *E. coli* is added to the lipid A core as an O polysaccharide [4], we analyzed the polysaccharides produced on the lipid A cores of seven *E. coli* transformants with every combination of *rgpE*, *rgpH*, and *rgpI* using ELISA with serotype c-specific and rhamnan-specific antisera (Table 2). The serotype c-specific antiserum reacted with the RGPs of KD602, KD604, KD606, and KD607, but not with the RGPs of KD601, KD603, and KD605. These data indicate that RgpH alone produces RGP that contains glucose side-chains, and RgpE and RgpI are not essential for glucose side-chain formation in *E. coli*.

On the other hand, the RGP of each of the *E. coli* transformants reacted with the rhamnan-specific antiserum, although the *S. mutans* RGP has strict specificity for the serotype c-specific antiserum. This means that the RGPs produced by the *E. coli* transformants were not completed with glucose side-chain formation. Furthermore, the RGP reactivities with rhamnan-specific antisera of KD606 and KD607 were approximately 50% of those from KD602 and KD604. The ratios of reactivity with rhamnan-specific antiserum to reactivity with serotype c-specific antiserum were 0.33, 0.34, 0.15, and 0.14 for the RGPs from KD602, KD604, KD606, and KD607, respectively. Analysis by ANOVA, with the Bonferroni multiple-comparison test, revealed that there were significant differences in reactivity ratios between KD602 and KD606, KD602 and KD607, KD604 and KD606, and KD604 and KD607 ($P < 0.01$). The *rgpI* gene, only in the presence of *rgpH*, increased the reactivity of RGP with serotype c-specific antiserum, and decreased reactivity with rhamnan-specific antiserum. Considering that the α1,3-glucosyl residue is the immunological determinant of the serotype c antigen, the increase in reactivity with serotype c-specific antiserum and the decrease in reactivity with rhamnan-specific antiserum may reflect differences in the frequency of glucose side-chains on the rhamnan backbones. These results indicated that RgpI might exert positive control over RgpH-mediated side-chain formation.

In contrast, KD601 harboring *rgpE* did not produce RGP that reacted with serotype c-specific antiserum, and there were no significant differences in immunoreactivity between either the RGPs of KD602 and KD604 or those of KD606 and KD607 (Table 2). These results suggest that RgpE does not function in RGP synthesis in *E. coli* strain, despite the fact that *rgpE* is involved in glucose side-chain formation in RGP synthesis in *S. mutans* [3].

3.4. Chemical analysis of RGPs from KD604 and KD607

To confirm the role of RgpI, the sugar compositions of the RGPs from KD604 and KD607 were analyzed by HPLC. The ratio of rhamnose to glucose in the RGP of KD607 was 3.4, whereas the corresponding ratio for the RGP of KD604 was 4.7 (Table 3). This result was consistent with the immunoreactivities of the RGPs from these transformants and reinforced our hypothesis that *rgpI* is involved in the control of glucose branching on the rhamnan backbone. However, the ratio of rhamnose to glucose in the RGP of KD607 was higher than that (nearly 2) of the RGP from *S. mutans* Xc. It is possible that an additional gene is required for the accomplishment of glucose side-chain formation, or that *rgpE* does not work well in *E. coli*.

3.5. Effect of RgpI on the expression of rgpH

We considered the possibility that RgpI affected the transcriptional levels of *rgpH* relative to those of the genes involved in rhamnan backbone synthesis (*rgpA* to *rgpF*, but excluding *rgpE*). To analyze the transcriptional levels, RNA dot blotting of the *rgpH*, *rgpA*, and *rgpF* genes was performed. No distinct differences were observed in the amounts of *rgpH* mRNA between KD604 and KD607, nor in the amounts of *rgpA* and *rgpF* mRNA (Fig. 2). These data indicated that RgpI did not affect the transcriptional level of *rgpH* compared with those of *rgpA* and *rgpF*, suggesting that the increased frequency of glucose side-chains on the RGP of KD607 was not due to changes in the transcription of *rgpH* relative to those of the genes involved in rhamnan backbone synthesis. However, in the present study, we did not directly confirm changes in the transcriptional levels of *rgpB*, *rgpC*,

Table 3
Sugar compositions of RGPs from KD604 and KD607

Strain	Approximate molar ratio of rhamnose to glucose	
	Rhamnose	Glucose
KD604	4.7	1.0
KD607	3.4	1.0

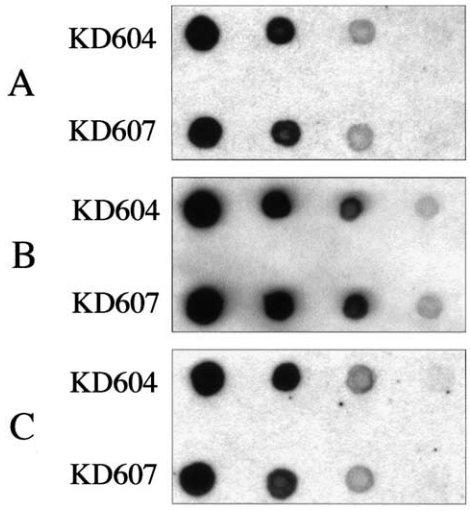

Fig. 2. Dot blot analysis of mRNA expression in KD604 and KD607. KD607 harbors *rgpI*, but KD604 does not. Five-fold serial dilutions of total RNA, starting with 10 μg, were applied to the membrane and hybridized with the *rgpA* (A), *rgpF* (B), or *rgpH* (C) probe. DNA probes were generated by amplifying 500-bp internal fragments of the *rgpA*, *rgpF*, and *rgpH* genes, and labeled with DIG by PCR. The locations of these probes are indicated in Fig. 1.

and *rgpD*. These genes, along with *rgpA* and *rgpF*, are located close to each other, and polycistronic transcription of the *rgpA* to *rgpF* region has been suggested. To confirm this fact, RT-PCR of mRNA from the *E. coli* strain harboring pRGP1 was performed with the RT1 primer (5′-GGCAATGCTTCCCAATGCTC-3′) to synthesize cDNA in the *rgp* region and the following primer sets: RT2F (5′-ATACGGACTTGAATCTTGT-3′) and RT2R (5′-CGACTAAAAAAGTCCATT-3′); RT3F (5′-CAGATTTGACAGTAGTAG-3′) and RT3R (5′-TCAAGGAAAAGCGGAATC-3′); RT4F (5′-TATTGCTACTCGCAGTGT-3′) and RT4R (5′-GCCGCTATTCTTTCTTTC-3′); RT5F (5′-CTGCCAGTGGACTTATTC-3′) and RT5R (5′-TCGGGAGCTATAAAATAA-3′); and RT6F (5′-GCATTCGGGCAATATTCAATT-3′) and RT6R (5′-CTTTTGAACATCCTGATACTG-3′), as shown in Fig. 1. The result of RT-PCR revealed that the *rgpA* to *rgpF* region constitutes one polycistronic operon in *E. coli* (data not shown). We conclude that RgpI regulates glucose side-chain formation on the rhamnan backbone in a positive fashion by altering the kinetics of RgpH, without affecting the transcriptional levels of the other genes involved in RGP synthesis.

Certain oligosaccharide or polysaccharide structures are critical for functionality. Leishmania parasites, which cause leishmaniasis, are equipped with cell surface lipophosphoglycan mainly composed of a backbone of alternating α1,6-linked galactoses and β1,4-linked mannoses and side-chains of β1,3-linked glucose. Although the regulation of side-chain formation in lipophosphoglycan is important for mediating specific host–parasite interactions, its mechanism remains unknown [15]. It has been previously reported that polysaccharide synthesis is regulated by proteins that derive from two-component system-dependent or -independent genes, in which the regulation of polysaccharide synthesis genes occurs at the transcriptional level [16–20]. However, the RgpI protein appears to regulate branching frequency in a manner that is different from the mechanisms described above.

Along with previous results, our finding that *rgpE* is involved in glucose side-chain formation in *S. mutans* suggests that at least three genes participate in the transfer of a glucose residue to the rhamnan backbone. Bearing in mind the consensus concept for glycosyl transfer, which proposes that a sole enzyme corresponds to a specific glycosyl transfer activity, we are currently unable to confirm the possibility that a combination of the functions of all three gene products contributes to α1,3-linked glucose side-chain formation on the rhamnan backbone. Much remains to be learnt about this enzymatic activity, and further characterization of the gene products is required to elucidate this unique glucosyl transfer mechanism.

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